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Saliva derived host defense peptides histatin1 and LL–37 increase secretion of antimicrobial skin and oral mucosa chemokine CCL20 in an IL–1α independent manner

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Submitted

Abstract

Even though skin and oral mucosa are continuously in contact with commensal and opportunistic microorganisms, they generally remain healthy and uninflamed. Host defense peptides (HDP) make up the body's first line of defense against many invading pathogens, and have been reported to be are involved in the orchestration of innate immunity and the inflammatory response. HDP are produced by a variety of immune- and epithelial cells and are present in saliva. In this study we investigated the effect of two salivary HDPs, LL-37 and Hst1, on the inflammatory and antimicrobial response by skin and oral mucosa (gingiva) keratinocytes and fibroblasts. The potent antimicrobial chemokine CCL20 was investigated and compared with chemokines CCL2, CXCL1, CXCL8 and CCL27 and pro-inflammatory cytokines IL-1α and IL-6. Keratinocyte-fibroblast co-cultures showed a synergistic increase in CCL20 secretion upon Hst1 and LL-37 exposure compared to mono-cultures. These co-cultures also showed increased IL-6, CXCL1, CXCL8 and CCL2 secretion, which was IL-1α dependent. Secretion of the antimicrobial chemokine CCL20 was clearly IL-1α independent, and since neither TNF-α, CCL27, CCL28 and IL-18 were responsible for the Hst1 and LL-37 increased CCL20 secretion, the soluble mediator is as yet unknown. These results indicate that salivary peptides have indirect host defense properties, since they can stimulate skin and gingiva to secrete antimicrobial chemokines. The fact that fibroblasts are indirectly triggered by salivary peptides to secrete the antimicrobial factor CCL20 may be part of the hosts defense to counteract deep dermal and lamina propria infection.

Introduction

Even though skin and oral mucosa are continuously in contact with commensal and opportunistic microorganisms, they generally remain healthy and uninflamed. Host defense peptides (HDP) make up the body's first line of defense against many invading pathogens, including bacteria, fungi and viruses. HDP are produced by a variety of immune- and epithelial cells and are present in a number of bodily fluids, including saliva [1;2]. HDPs have a direct antimicrobial function, because they can damage and kill microorganisms in multiple ways [3]. They can either form transmembrane pores, or translocate to the cytoplasm, where they have intracellular targets (e.g. alter cytoplasmic membrane septum formation, inhibit cell-wall, nucleic acid or protein synthesis, or inhibit enzymatic activity). In addition, HDP have also been reported to be involved in the orchestration of innate immunity and the inflammatory responses such as chemoattraction, wound healing, modulation of pro- and anti-inflammatory responses and cellular differentiation [1].

A well-described HDP is LL-37, which is part of the only human cathelicidin and named after its 37 amino acids sequence starting with two leucines. Healthy skin and gingiva secrete low amounts of LL-37, but upon injury or infection large amounts are released into the local environment by degranulating neutrophils and keratinocytes [1;4;5]. Besides its direct antimicrobial properties, LL-37 plays a central role in innate immune responses and inflammation, as it is a potent chemoattractant for monocytes, T-lymphocytes and neutrophils [6]. LL-37 also promotes wound healing in a concentration dependent manner [7]. At low concentrations it enhances fibroblast migration and keratinocyte proliferation and migration [8-10]. Since LL-37 suppresses collagen synthesis it also has anti-fibrotic activity, thus improving wound healing [11].

Another class of HDP that have been reported to have potent antimicrobial properties are histatins (Hst); in particular Hst3 and Hst5 [12;13]. Histatins are a family of peptides which are specifically secreted into the saliva of higher primates only. We have previously shown that histatins (Hst1 and Hst2) are the main factors in human saliva responsible for skin and oral keratinocyte and fibroblast migration, suggesting a role in wound closure [14-16]. Hst1 is also able to enhance cell-substrate adhesion and cell-cell interaction [17;18].

In addition to HDP, a number of chemokines which were originally described as being key players orchestrating cell trafficking throughout the body, have also been reported to have antimicrobial activity [19]. CCL20 is such a chemokine, originally identified as a chemoattractant to facilitate recruitment of CCR6-expressing cells, including memory T-cells, immature dendritic cells and T-helper 17 cells [20;21]. Interestingly, its only receptor (CCR6) is also the receptor for binding of human β-defensin 1 and 2 [22]. CCL20 has direct antimicrobial activity against many bacterial pathogens e.g. *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Enterococcus faecium, Pseudomonas aeruginosa, Moraxella catarrhalis and also against the yeast Candida albicans and Vaccinia virus* [23-25]. Similar to CCL20, CXCL1 has been reported to have direct antimicrobial activities, whereas CXCL8 exerts its full antimicrobial activities after proteolytic processing [23;24;26;27].

In this study we investigated the effect of two HDPs, LL-37 and Hst1, on the inflammatory and antimicrobial response by skin and oral mucosa (gingiva) cells (keratinocytes and fibroblasts). The potent antimicrobial chemokine CCL20 was investigated and compared with chemokines CCL2, CXCL1, CXCL8 and CCL27 and pro-inflammatory cytokines IL-1α and IL-6.

Material and methods

Human skin and gingiva culture

Human abdominal skin was obtained after informed consent from patients undergoing corrective abdominal plastic surgery; gingiva was obtained after informed consent from healthy donors after molar tooth extraction or dental implant surgery. Tissue was used in an anonymous fashion in accordance with the "Code for Proper Use of Human Tissues" as formulated by the Dutch Federation of Medical Scientific Organizations (www.fmwv.nl) and following procedures approved by the institutional review board of the VU University medical centre, Amsterdam, The Netherlands.

Monocultures and co–culture of fibroblasts and keratinocytes

Keratinocytes and fibroblasts were isolated from skin and gingiva tissue and cultured as described earlier [28]. Fibroblasts (passage 3) were seeded as a monoculture at a density of 7 x 10^3 cell/cm^2 in 6-well culture plates in fibroblast medium, consisting of Dulbecco's modified Eagle medium (DMEM) (Lonza, Verviers, Belgium) containing 1% ultroserG (UG) (Biosepra, Cergy-Saint-Christophe, France) and 1% penicillin-streptomycin (P/S) (Gibco). Keratinocytes (passage 2) were seeded as a monoculture at a density of 4 x 10^4 cells/cm^2 in keratinocyte medium in 6-well culture plates, pre-coated with 0.5 µg/cm^2 human placental collagen IV (Sigma-Aldrich). Keratinocyte medium consisted of DMEM/Ham's F-12 (Gibco) (3:1), 1% UG, 1% P/S, 1µM isoproterenol (Sigma-Aldrich), 0.1 µM insulin (Sigma-Aldrich). For co-cultures, first the fibroblasts were seeded at a density of 7 x 10^3/cm^2 on collagen IV coated 6-well plates in fibroblast medium. After initial attachment of fibroblasts (4 h) the keratinocytes were seeded in the same 6-well plates at a density of 2.4 x 10^3 cell/cm^2 in keratinocyte medium. This results in a well with 75% fibroblasts and 25% keratinocytes. After initial attachment of keratinocytes (4 h), medium was switched to keratinocyte medium.

Peptide synthesis

Hst1 (DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN) and LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) were synthesized by solid-phase peptides synthesis using Fmoc chemistry with a Syro II synthesizer (Biotage, Uppsala, Sweden). Purification was conducted by ultimate 3000 RP-HPLC (Thermo Scientific) and authenticity was confirmed by mass spectrometry (MALDI-TOF) (Bruker Daltonik GmbH Germany) as previously described [29].

Histatin1 and LL–37 exposure

After overnight attachment, the cells were supplemented with Hst1 (2, 4 and 50 µM) or LL-37 (2, 4 and 10 µM), or vehicle (H_2O) as negative control. TNF-α (Miltenyi biotech, cat nr 130-094-014) (10 ng/ml) was used as positive control in fibroblast experiments. After 24 h of exposure the supernatant was collected and stored at -20˚C until further analysis by ELISA. The attached cells were used to determine cell viability after exposure by MTT analysis. The 6-well plates were washed with PBS before addition of 2 mg/ml MTT solution (Sigma-Aldrich) and incubated at 37˚C for 2 h. After that, the MTT solution was removed and 2-propanol was added. Color intensity was measured at 570 nm in a spectrophotometer.

For the crossover experiments fibroblasts and keratinocyte mono-cultures were cultured as described above. Instead of exposure to different concentrations of Hst1 and LL-37, the fibroblasts were exposed to 10% (v/v) of the 24 h supernatant of exposed keratinocytes in 90% (v/v) fibroblast medium and the keratinocytes to 10% (v/v) of the 24 h supernatant of exposed fibroblasts in 90% (v/v) keratinocyte medium for 24 h.

Exposure to Hst1 and LL–37 with addition of IL–1α, TNFα, CCL27, CCL28 and IL–18 neutralizing antibodies

The cells were seeded in exactly the same way as described above for co-culture experiments. After overnight attachment of the cells, neutralizing antibodies or isotype controls (100 ng/ml) were added to the cultures as recommended by the supplier. Neutralizing antibodies IL-1α (R&D: AF-200-NA), TNFα (R&D: AF-210-NA), CCL27 (R&D: AF-376), CCL28 (R&D: AF-717) all had Goat IgG (R&D: AB-108-C) as isotype control. For IL-18 (R&D: D044-3) a mouse IgG1 (R&D: MAB002) was used as isotype control. After 30 min the culture medium was further supplemented with Hst1 (4 and 50 µM), LL-37 (2 and 4 µM) or vehicle (H2O). 24 h after exposure the supernatant was collected and stored at -20˚C until further analysis by ELISA and cell viability was determined using MTT assay (as described above).

ELISA

For IL-6, CCL2, CCL20, CCL27, CXCL1 and IL-1α quantification in culture supernatant, ELISA reagents were used in accordance with the manufacturer's specifications. These cytokines were measured by paired ELISA antibodies and recombinant proteins obtained from R&D Systems Inc. (Minneapolis, Minnesota, USA). CXCL8 was measured by a Pelipair reagent set (Sanquin, Amsterdam, the Netherlands).

Statistics

All data are presented as mean ± standard error mean. Differences in the monocultures of fibroblasts and keratinocytes, as well as the co-cultures exposed to Hst1 and LL-37 were compared with vehicle (H_2O) by repeated measures one-way ANOVA with Dunnett's multiple comparisons test. The differences in co-cultures of fibroblasts and keratinocytes exposed to

Hst1 and LL-37 with IL-1α neutralizing antibodies were compared with isotype (goat IgG) by repeated measures one-way ANOVA with Bonferoni's multiple comparisons test. Statistics were calculated in Graphpad Prism (San Diego, CA, USA). Differences were considered significant when *P < 0.05, **P < 0.01, ***P <0.005.

Results

Hst1 and LL–37 stimulate keratinocytes, but not fibroblasts to secrete CCL20

In order to determine whether Hst1 and LL-37 could stimulate secretion of the chemokine CCL20 by skin and gingiva cells, keratinocyte and fibroblast mono-cultures, as well as co-cultures of both cell types were exposed to Hst1 and LL-37. Whereas Hst1 was not cytotoxic at concentrations up to 50 μM, LL-37 was extremely cytotoxic at 50 μM and therefore not further investigated. All other Hst1 and LL-37 concentrations used in this study resulted in less than 30% cytotoxicity with the exception of 10 μM of LL-37, which resulted in up to 70% cytotoxicity in some experimental conditions (Figure 1).

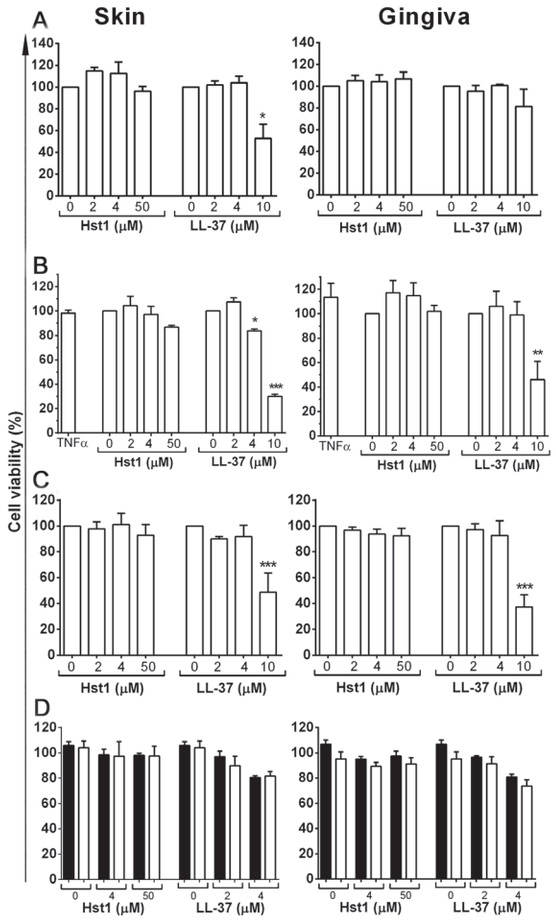

Figure 1: Cell viability after exposure to Hst1 and LL-37

Cell viability is shown after 24 hour exposure to Hst1 and LL-37 of A: keratinocyte mono-layer, B: fibroblast monolayer, C: keratinocyte-fibroblast co-culture and D: keratinocyte-fibroblast co-culture with IL-1α neutralizing antibodies (white bars), or isotype control (black bars). Each bar represents the mean ± standard error mean of 3 independent experiments each performed in duplicate, except D (N=4).

Exposure to Hst1 or LL-37 resulted in a dose dependent increase in CCL20 from both skin and gingiva keratinocytes, with LL-37 being more potent than Hst1. When exposed to 50 μM Hst1, skin and gingiva keratinocytes showed respectively a 3.5-fold and 1.5-fold increase in secretion of CCL20 (Figure 2A). Exposure to LL-37 (2, 4 and 10 μM) resulted in a 10-fold increase of CCL20 secretion by skin keratinocytes and 2-fold increase by gingiva keratinocytes. In contrast, Hst1 and LL-37 exposure did not induce CCL20 secretion by fibroblasts (Figure 2B), whereas TNFα, the positive control, increased CCL20 secretion by both skin and gingiva fibroblasts.

To investigate the effect of cross-talk between keratinocytes and fibroblasts on Hst1 or LL-37 mediated CCL20 secretion, co-culture experiments were performed. Skin-derived keratinocyte-fibroblast co-cultures exposed to 50 μM Hst1 showed 12-fold increase in CCL20 secretion, and gingiva co-cultures showed >3-fold increase. Exposure to 10 μM LL-37 resulted in >75-fold increase in CCL20 secretion from skin co-cultures, and >40-fold increases secretion from gingiva co-cultures (Figure 2C). Since the amount of cells in the mono-cultures and co-cultures is different, the absolute secretion of CCL20 cannot be compared, therefore the fold-increase of the exposed cultures compared to the unexposed cultures was compared. The fold increase for co-cultures was clearly greater than that observed for mono-cultures, indicating a synergistic cross-talk has occurred between soluble mediators secreted by keratinocytes and/or fibroblasts.

Hst1 and LL–37 exposed keratinocytes stimulate CCL20 secretion by fibroblasts in an IL–1α, TNF–α, IL–18, CCL27, CCL28 independent manner

Since synergism occurred with regards to CCL20 secretion in response to Hst1 and LL-37, we next determined whether soluble mediators were involved (rather than direct cell-cell contact) and whether the fibroblasts triggered keratinocytes to secrete CCL20 or vice versa (Figure 2D). Fibroblasts exposed to 10% culture supernatant derived from keratinocytes treated with 50 μM Hst1, showed aproximately 3-fold increase in CCL20 secretion. Similar experiments with culture supernatant derived from keratinocytes treated with 2 or 4 μM LL-37 resulted in >5-fold and >8 fold increase in CCL20 secretion by fibroblasts respectively. Similar to skin, exposure of gingiva fibroblasts to culture supernatant derived from LL-37 exposed gingiva keratinocytes resulted in 2-fold increase with the 2 μM LL-37 condition and 6-fold for 4 μM LL-37 condition (Figure 2D). Notably, CCL20 secretion by keratinocytes was not stimulated by culture supernatant derived from Hst1 or LL-37 treated skin and gingiva fibroblasts. Taken together these results suggest that upon exposure to Hst1 or LL-37 keratinocytes secrete a soluble mediator which can trigger fibroblasts to secrete CCL20.

Since IL-1α has been reported to increase CCL20 secretion by keratinocytes and fibroblasts [30;31] we next determined whether IL-1α could be the soluble mediator secreted by keratinocytes after exposure to Hst1 or LL-37 resulting in CCL20 secretion by fibroblasts. Addition of neutralizing IL-1α antibodies to the culture medium before exposure resulted only in a slight reduction (~15%) in the increased CCL20 secretion after LL-37 exposure, but not Hst1 exposure, both from skin and gingiva co-cultures (Figure 2E). Since IL-1α was clearly not identified as the keratinocyte derived soluble mediator, similar experiments were performed with antibodies against other keratinocyte

derived pro-inflammatory cytokines TNF-α, CCL27, CCL28 and IL-18 (data not shown). However, similar to IL-1α, neutralizing antibodies to these cytokines had no effect on CCL20 secretion by skin and gingiva co-cultures. These results indicate that Hst1 and LL-37 increase CCL20 secretion in an IL-1α, TNF-α, CCL27, CCL28 and IL-18 independent manner.

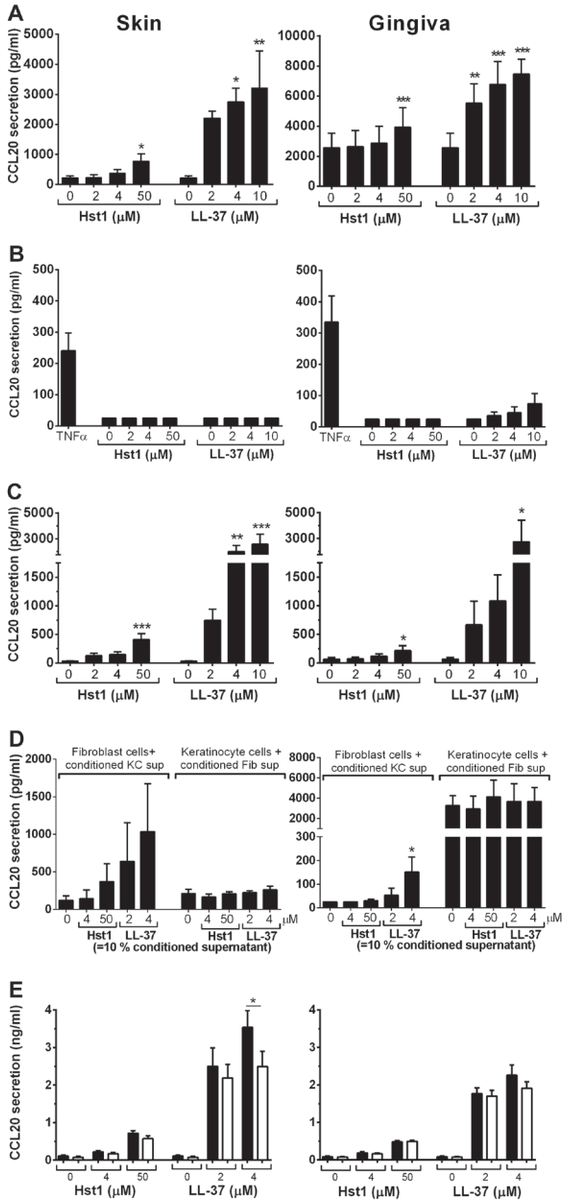

Figure 2: CCL20 secretion after Hst1 and LL-37 exposure

Skin or gingiva cells were exposed to either Hst1 or LL-37 for 24 hours and CCL20 secretion was assessed by ELISA. A: keratinocyte mono-culture, B: fibroblast mono-culture and C: keratinocyte-fibroblast co-culture. D: crossover experiments with fibroblast mono-culture (left) exposed to 10% culture supernatant from keratinocyte mono-cultures that were exposed to Hst1 and LL-37 (KC sup), and keratinocyte mono-culture (right) exposed to 10% culture supernatant from fibroblast mono-cultures that were exposed to Hst1 and LL-37 (Fib sup). E: keratinocyte-fibroblast co-culture exposed to Hst1 and LL-37, together with either IL-1α neutralizing antibodies (white bars), or isotype control (black bars). Each bar represents the mean ± standard error mean of 3 independent experiments each performed in duplicate, except C (N=4) and (D, N=5 for the skin fibroblasts exposed to 10% supernatant of exposed skin keratinocytes).

Hst1 and LL–37 mediated secretion of inflammatory mediators

Next we determined whether the results obtained for CCL20 were typical for other inflammatory and antimicrobial cytokines and chemokines. Secretion of keratinocyte derived inflammatory mediators IL-1α and CCL27 and fibroblast derived inflammatory (IL-6, CXCL8) and antimicrobial mediators (CXCL1, CXCL8, CCL2) were investigated in mono-cultures (Figure 3). Hst1 was unable to increase IL-1α and CCL27 secretion by skin or gingiva

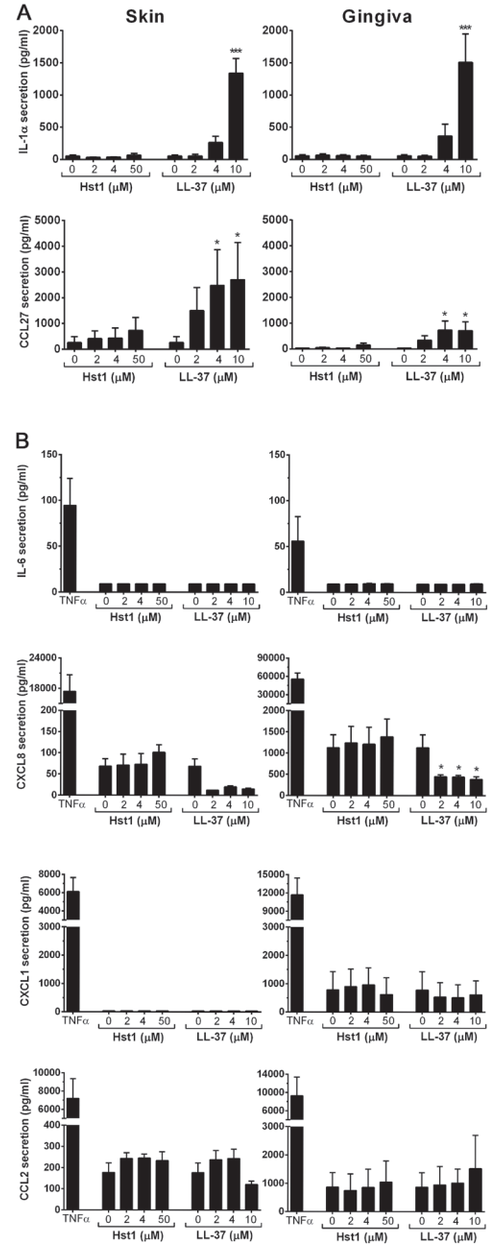

Figure 3: inflammatory cytokine secretion after Hst1 and LL-37 exposure

Skin or gingiva cells were exposed to either Hst1 or LL-37 for 24 hours and cytokine secretion was assessed by ELISA. A: IL-1α and CCL27 secretion by keratinocyte mono-cultures, B: IL-6, CCL2, CXCL1 and CXCL8 secretion by fibroblast mono-cultures. Each bar represents the mean ± standard error mean of 3 independent experiments each performed in duplicate.

keratinocytes (Figure 3A). However, when keratinocytes were exposed to 4 μM and 10 μM LL-37, IL-1α secretion increased approximately 5-fold and approximately 25-fold respectively. CCL27 secretion increased >10-fold after 4 and 10 μM LL-37 exposure. The stimulation of IL-1α secretion by gingiva keratinocytes was similar to that of skin keratinocytes, while CCL27 secretion was higher in skin than it was in gingiva, both in basal secretion as well as after Hst1 and LL-37 stimulation. The highest concentration of LL-37 tested (10 μM) had a negative effect on skin keratinocyte viability as tested with MTT assay (Figure 1). Hst1 did not affect keratinocyte viability even at concentrations as high as 50 μM.

Similar to our findings with CCL20, neither Hst1 nor LL-37 were able to increase IL-6, CXCL1, CXCL8 or CCL2 secretion by fibroblasts derived from skin or gingiva (Figure 3B). In fact, CXCL8 secretion by fibroblasts decreased by >2.5-fold for both skin and gingiva fibroblasts when exposed to LL-37, but not Hst1.

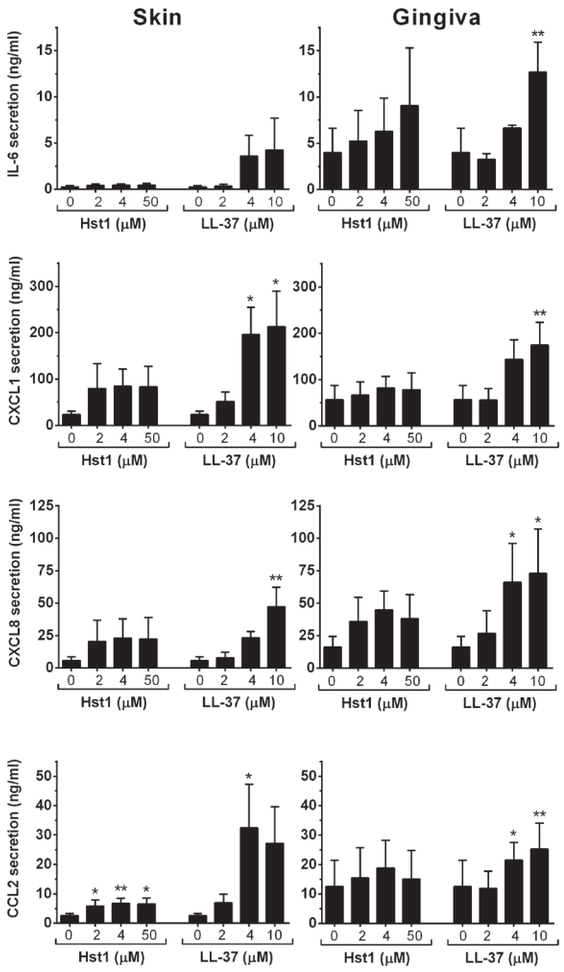

Figure 4: inflammatory cytokine secretion by keratinocyte-fibroblast co-cultures after Hst1 and LL-37 exposure
IL-6, CCL2, CXCL1 and CXCL8 secretion after 24 hours, by skin and gingiva keratinocyte-fibroblast co-cultures is shown. Each bar represents the mean ± standard error mean of 4 independent experiments each performed in duplicate.

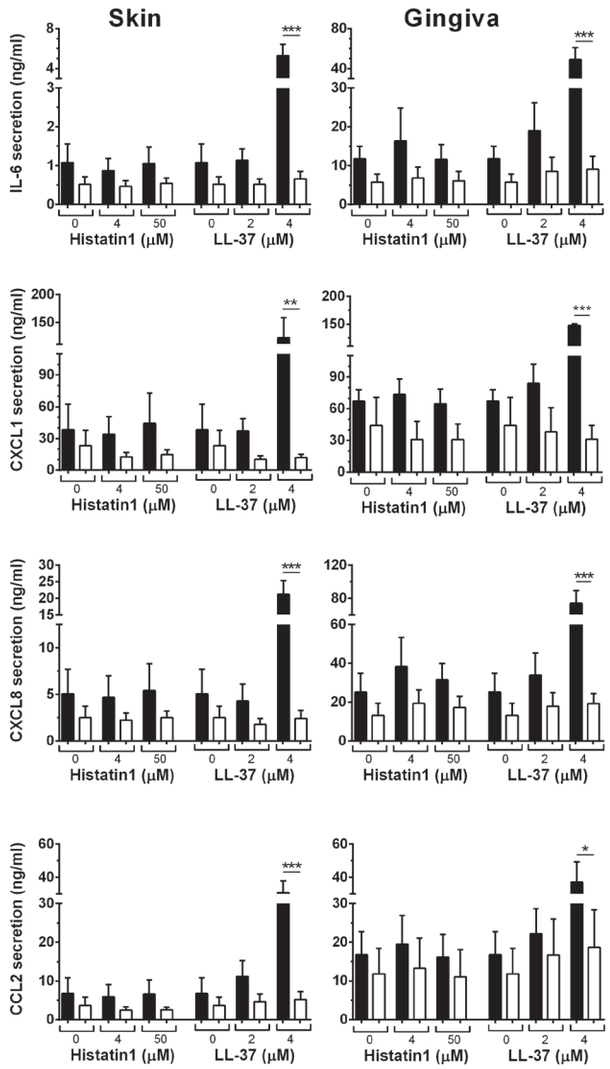

Figure 5: inflammatory cytokine secretion by keratinocyte-fibroblast co-cultures is blocked by IL-1α neutralizing antibodies
IL-6, CCL2, CXCL1 and CXCL8 secretion by skin and gingiva keratinocyte-fibroblast co-cultures after 24 hour exposure to Hst1 and LL-37, together with either IL-1α neutralizing antibodies (white bars) or isotype control (black bars). Each bar represents the mean ± standard error mean of 3 independent experiments each performed in duplicate.

Hst1 and LL–37 mediated secretion of inflammatory mediators, with the exception of CCL20, is IL–1α dependent

Since CCL20 secretion by fibroblasts grown in co-culture with keratinocytes occurred in an IL-1*α* independent manner, it was next determined whether this was also the case for other typical inflammatory or antimicrobial mediators (IL-6, CXCL1, CXCL8 and CCL2). Notably, LL-37 exposure resulted in a significant increase in all inflammatory mediators in both skin and gingiva co-cultures (Figure 4), which was totally blocked by incubation with neutralizing

antibodies to IL-1α to values similar to basal secretion (Figure 5). In contrast, Hst1 exposure resulted in slight trends for increased secretion of the inflammatory mediators with only significance occurring for CCL2 secretion. However, in all cases neutralizing IL-1α antibodies reduced secretion of these inflammatory mediators to values similar to basal secretion. Taken together, these results show that in contrast to CCL20 secretion, the secretion of IL-6, CXCL1, CXCL8 and CCL2 induced by Hst1 or LL-37 is mediated by IL-1α.

Discussion

In this study we have shown that peptides which are present in human saliva, Hst1 and LL-37, can stimulate host cells (skin and gingiva fibroblasts and keratinocytes) to secrete known antimicrobial and inflammatory mediators (CCL20, IL-1α, IL-6, CCL2, CCL27, CXCL1 and CXCL8) [23-25]. This suggests that these HDPs, in addition to having direct antimicrobial properties, also have indirect antimicrobial properties by stimulating a host antimicrobial response.

We found that Hst1 and LL-37 stimulated keratinocytes to secrete a soluble mediator, which was required for fibroblasts to secrete the cytokines and chemokines studied. Fibroblasts were unable to directly respond to Hst1 and LL-37. For IL-6, CCL2, CXCL1 and CXCL8, the keratinocyte derived soluble mediator was IL-1α, since neutralizing antibodies to IL-1α could totally block cytokine secretion to baseline levels or even below baseline levels. However, for CCL20, the soluble mediator was not IL-1α.. These findings were particularly surprising since we and others have reported that CCL20 can be secreted by keratinocytes and human skin equivalents in an IL-1α dependent manner [30;31] and that the contact allergen nickel sulfate and the contact irritant sodium dodecyl sulfate (SDS) increase CCL20 secretion from human skin equivalents in an IL-1α dependent manner [31]. The soluble mediator for the increased CCL20 secretion by keratinocyte-fibroblast co-cultures was also not another keratinocyte pro-inflammatory cytokine, such as TNF-α, IL-18 or CCL27. Therefore the soluble mediator is as yet still unknown. It is described that incubation of keratinocytes with LL-37 increased CCL20 secretion, and this was decreased by addition of pertussis toxin, indicating that a G-protein-coupled receptor might be involved [32].

Keratinocyte cultures, in contrast to fibroblast cultures, were able to increase the amount of CCL20 secretion directly in response to Hst1 or LL-37. This may be due to an autocrine feedback mechanism via the as yet unknown soluble mediator [32;33].

Previously we reported that CCL20, in contrast to CCL27, CXCL1 and CXCL8, does not increase keratinocyte migration or proliferation, even though it is produced by keratinocytes from excised skin and epidermal equivalents and its secretion was increased upon freeze-wounding of epidermal equivalents [33]. From these findings and our current findings, we can conclude that a role of CCL20 is probably to control pathogen infection after wounding, rather than wound closure. This is supported by others who have shown that CCL20 has direct antimicrobial activity against many bacterial pathogens e.g. *E. coli, S. aureus, S. pyogenes, E.*

faecium, P. aeruginosa, M. catarrhalis and also *C albicans and Vaccinia virus* [23-25]. Notably, *S. aureus, E. coli* and *P. aeruginosa* have been reported to be present in chronic wounds [34-40].

Taken together, our results show that Hst1 and LL-37 can stimulate host cells to secrete antimicrobial CCL20 via an as yet unknown mechanism. LL-37 is thought to alter signaling pathways in the host cell, triggering a (cytotoxic) immune response [1]. However, the receptor for histatins is still unknown. The fact that fibroblasts are indirectly triggered by salivary peptides to secrete the antimicrobial factor CCL20 may be part of the hosts defense to counteract deep dermal and lamina propria infection.

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